TITLE: **TPS PLANT GENE CONSTRUCTS AND TRANSFORMANTS**

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TPS PLANT GENE CONSTRUCTS AND TRANSFORMANTS

[0001] This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/424,410, filed November 6, 2002, and U.S. Provisional Patent Application Serial No. 60/430,861, filed December 4, 2002.

FIELD OF THE INVENTION

[0002] The present invention relates to transgenic monocot plants which are transformed with a nucleic acid encoding an enzyme in the trehalose biosynthetic pathway to increase tolerance to low temperature stress, water stress, and salt stress.

BACKGROUND OF THE INVENTION

[0003] The explosive increase in world population, along with the continuing deterioration of arable land, scarcity of fresh water, and increasing environmental stress pose serious threats to global agricultural production and food security. Despite focused efforts to improve major crops for resistance to abiotic stresses such as drought, excessive salinity, and low temperature by traditional breeding, success has been limited (Boyer, J. S., "Plant Productivity and Environment," *Science*, 218:443-448 (1982)). This lack of desirable progress is attributable to the fact that tolerance to abiotic stress is a complex trait that is influenced by coordinated and differential expression of a network of genes. Fortunately, it is now possible to use transgenic approaches to improve abiotic stress tolerance in agriculturally important crops with far fewer target traits than had been anticipated (Zhang et al., "Engineering Salt-Tolerant *Brassica* Plants: Characterization of Yield and Seed Oil Quality in Transgenic Plants with Increased Vacuolar Sodium Accumulation," *Proc. Natl. Acad. Sci. USA*, 98:12832-12836 (2001)).

[0004] Abiotic stresses can directly or indirectly affect the physiological status of an organism by altering its metabolism, growth, and development. A common response of organisms to drought, salinity, and low-temperature stresses is the accumulation of sugars and other compatible solutes (Hare et al., "Dissecting the

Roles of Osmolyte Accumulation During Stress," *Plant Cell Environ.*, 21:535-553 (1998)). These compounds serve as osmoprotectants and, in some cases, stabilize biomolecules under stress conditions (Hare et al., "Dissecting the Roles of Osmolyte Accumulation During Stress," *Plant Cell Environ.*, 21:535-553 (1998); Yancey et al., "Living with Water Stress: Evolution of Osmolyte Systems," *Science*, 217:1214-1222 (1982)). One such compound is trehalose, a nonreducing disaccharide of glucose, which plays an important physiological role as an abiotic stress protectant in a large number of organisms, including bacteria, yeast, and invertebrates (Crowe et al., "Anhydrobiosis," *Annu. Rev. Physiol.*, 54:579-599 (1992)). Trehalose has been shown to stabilize dehydrated enzymes, proteins, and lipid membranes efficiently, as well as protect biological structures from damage during desiccation. In the plant kingdom, most species do not seem to accumulate detectable amounts of trehalose, with the notable exception of the highly desiccation-tolerant "resurrection plants" (Wingler, "The Function of Trehalose Biosynthesis in Plants," *Phytochemistry*, 60:437-440 (2002)). The recent discovery of homologous genes for trehalose biosynthesis in *Selaginella lepidophylla, Arabidopsis thaliana*, and several crop plants suggests that the ability to synthesize trehalose may be widely distributed in the plant kingdom (Goddijn et al., "Trehalose Metabolism in Plants," *Trends Plant Sci.*, 4:315-319 (1999)). A putative plant gene for trehalose-6-phosphate synthase (TPS) can complement a *Δtps1* mutant yeast strain, suggesting that the plant and yeast gene products are functionally similar (Zentella et al., "A *Selaginella lepidophylla* Trehalose-6-Phosphate Synthase Complements Growth and Stress-Tolerance Defects in a Yeast *tps1* Mutant," *Plant Physiol.*, 119:1473-1482 (1999)).

[0005] In bacteria and yeast, trehalose is synthesized in a two-step process: trehalose-6-phosphate is first formed from UDP-glucose and glucose-6-phosphate in a reaction catalyzed by TPS. Trehalose-6-phosphate is then converted to trehalose by trehalose-6-phosphate phosphatase (TPP) (Goddijn et al., "Trehalose Metabolism in Plants," *Trends Plant Sci.*, 4:315-319 (1999)). Metabolic engineering for enhanced accumulation of trehalose in plants has been the recent focus of attention in some model dicot plants (Holmstrom et al., "Drought Tolerance in Tobacco," *Nature*, 379:683-684 (1996); Goddijn et al., "Inhibition of Trehalase Activity Enhances Trehalose Accumulation in Transgenic Plants," *Plant Physiol.*, 113:181-190 (1997);

Romero et al., "Expression of the Yeast Trehalose-6-Phosphate Synthase Gene in Transgenic Tobacco Plants: Pleiotropic Phenotypes Include Drought Tolerance," *Planta*, 201:293-297 (1997); Pilon-Smits et al., "Trehalose-Producing Transgenic Tobacco Plants Show Improved Growth Performance Under Drought Stress," *J. Plant Physiol.*, 152:525-532 (1998)). However, in these previous studies, constitutive overexpression of TPS and/or TPP genes from yeast or *Escherichia coli* in tobacco or potato plants resulted in undesirable pleiotropic effects, including stunted growth and altered metabolism under normal growth conditions (Goddijn et al., "Inhibition of Trehalase Activity Enhances Trehalose Accumulation in Transgenic Plants," *Plant Physiol.*, 113:181-190 (1997); Romero et al., "Expression of the Yeast Trehalose-6-Phosphate Synthase Gene in Transgenic Tobacco Plants: Pleiotropic Phenotypes Include Drought Tolerance," *Planta*, 201:293-297 (1997); Pilon-Smits et al., "Trehalose-Producing Transgenic Tobacco Plants Show Improved Growth Performance Under Drought Stress," *J. Plant Physiol.*, 152:525-532 (1998)). The present invention is directed to producing transgenic monocot plants with improved low temperature stress, water stress, and salt stress tolerance.

SUMMARY OF THE INVENTION

[0006] The present invention relates to a transgenic monocot plant transformed with a nucleic acid encoding an enzyme for trehalose biosynthesis, under the control of an inducible promoter, that confers low temperature, salt, and water stress tolerance to a monocot plant.

[0007] The present invention further relates to a monocot plant cell or protoplast transformed with a nucleic acid encoding an enzyme for trehalose biosynthesis, under control of an inducible promoter, that confers low temperature, salt, and water stress tolerance to a monocot plant regenerated from a monocot plant cell or protoplast.

[0008] The present invention also relates to a method of conferring tolerance to low temperature, salt, and water stress to a monocot plant by transforming a monocot plant cell or protoplast with a nucleic acid encoding an enzyme for trehalose biosynthesis, under control of an inducible promoter, under conditions effective to

impart low temperature, salt, and water stress tolerance to monocot plants regenerated from the monocot plant cell or protoplast.

[0009] Another aspect of the present invention further relates to a method of increasing tolerance of monocot plant to low temperature, salt, or water stress conditions by increasing the levels of an enzyme for trehalose biosynthesis in the monocot plant.

[0010] The present invention also relates to a transgenic monocot plant transformed with a plasmid that confers low temperature, salt, and water stress tolerance to the monocot plant where the plasmid comprises a first nucleic acid encoding trehalose-6-phosphate synthase, a first inducible promoter, the promoter located 5' to the first nucleic acid and controlling expression of the first nucleic acid, and a first termination sequence located 3' to the first nucleic acid.

[0011] Considering the importance of rice as a major crop, developing new cultivars with enhanced abiotic stress tolerance would undoubtedly have an enormous impact on global food production. It was decided to improve abiotic stress tolerance by transforming rice with a trehalose-6-phosphate synthase/phosphatase (*TPSP*) fusion gene that includes the coding regions of the *E. coli otsA* and *otsB* genes (encoding TPS and TPP, respectively) (Seo et al., "Characterization of a Bifunctional Enzyme Fusion of Trehalose-6-Phosphate Synthetase and Trehalose-6-Phosphate Phosphatase of *Escherichia coli*," *Appl. Environ. Microbiol.*, 66:2484-2490(2000)). This approach has the dual advantages of necessitating only a single transformation event and producing a higher net catalytic efficiency for trehalose formation (Seo et al., "Characterization of a Bifunctional Enzyme Fusion of Trehalose-6-Phosphate Synthetase and Trehalose-6-Phosphate Phosphatase of *Escherichia coli*," *Appl. Environ. Microbiol.*, 66:2484-2490(2000)). Because *indica* rice varieties represent 80% of rice grown worldwide, the economically valuable *indica* rice Pusa Basmati-1 (PB-1) was chosen to transform, even though transformation and regeneration are more difficult than in *japonica* rice varieties. Therefore, whatever has been accomplished with an *indica* rice works equally well with a *japonica* rice variety.

[0012] It was shown that engineering trehalose overproduction in rice can be achieved by stress-inducible or tissue-specific expression of bifunctional TPSP fusion enzyme without any detrimental effect on plant growth or grain yield. During abiotic

stress, transgenic plants accumulated increased amounts of trehalose and showed high levels of tolerance to salt, drought, and low-temperature stresses, as compared with the nontransformed plant. These results demonstrate the potential use of the transgenic approach in developing new rice cultivars with increased abiotic stress tolerance and enhanced rice productivity.

[0013] The present invention allows the production of monocot plants with increased tolerance to low temperature stress, salt stress and water stress (drought). In particular, increased tolerance in response to low temperature, salt, and water stress can be achieved by the activation of trehalose biosynthesis under the control of an inducible promoter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figures 1 *A* – *E* show a schematic representation of the expression vectors and DNA-blot hybridization analysis. Two binary plasmids, each containing the trehalose biosynthetic fusion gene (*TPSP*) that includes the coding regions of the *E. coli otsA* and *otsB* genes (encoding TPS and TPP, respectively), were constructed and transformed into *indica* rice. Figure 1 *A* shows the pSB109-TPSP plasmid. Figure 1 *B* shows the pSB-RTSP plasmid. Shaded boxes represent promoter elements (ABA, ABA-inducible; rbcS, rice *rbcS*; 35S, cauliflower mosaic virus 35S promoter); RB and LB represent T-DNA border on the right and left sides, respectively. Figure 1 *C* shows a more detailed schematic representation of pSB109-TPSP and pSB-RTSP including several restriction endonucleotide sites. Figure 1 shows a DNA-blot hybridization analysis from nontransformed control (NTC) plant, and representative transgenic plants of nine A-lines (Figure 1 *D*) and five R-lines (Figure 1 *E*) that were transformed with the plasmid pSB109-TPSP and pSB-RTSP, respectively. The rice genomic DNA was digested with *Hin*dIII (a unique site in the plasmid pSB109-TPSP, whereas two sites are present in the plasmid pSB-RTSP) and DNA blot hybridization analysis was performed with the 2.2-kb *TPSP* fusion gene as the probe. Molecular sizes (kb) are indicated.

[0015] Figures 2 *A* – *F* show the salt tolerance of rice plants and changes in mineral nutrition caused by salt stress. Figure 2 *A* shows plant roots after 4 weeks of

continuous 100 mM NaCl stress; the plants were not stressed in NTC. Figure 2 *B* shows dry weight of shoots (black bars) and roots (white bars) of plants grown under salt stress (NTS, R80, and A05) or no stress (NTC) conditions. Figure 2 *C* shows Western blots of leaf extracts (20 μg of proteins) immediately after salt stress of plants. (Figures 2 *D* -- *F*) Plant mineral nutrient content in shoots (black bars) and roots (white bars) under salt stress (NTS, R80, and A05) or no stress (NTC) conditions. Figure 2 *D* shows Na^+. Figure 2 *E* shows K^+. Figure 2 *F* shows Na^+/K^+ ratio. The ionic concentration is presented as mg/g dry weight. Values are the means ± SD ($n = 5$).

[0016] Figures 3 *A* – *D* show the appearance of plants and chlorophyll fluorescence parameters during drought stress. Five-week-old nontransformed and T_4 generation transgenic (R80 and A05) seedlings grown in soil were subjected to two cycles of 100 h of drought stress followed by watering for 3 weeks. Figure 3 *A* shows plants grown under well watered conditions (NTC, nontransgenic plants). Figure 3 *B* shows plants of the same age after two cycles of drought-stress treatment (NTS, nontransgenic plants after drought stress). Figures 3 *C* and *D* show chlorophyll fluorescence measurements on young, fully expanded leaves during the first cycle of 100 h of continuous drought stress. Figure 3 *C* shows ϕ_{PSII}, a measure of the efficiency of PS II photochemistry under ambient growth conditions. Figure 3 *D* shows decreases in Fv/Fm are a measure of photooxidative damage to PS II. ▲, nontransformed plants; ■, R80; ●, A05. Dotted lines represent the range of values for nonstressed control plants of all lines. Data represent means ± SD ($n = 5$) from independent plants.

[0017] Figure 4 shows trehalose content in shoots of transgenic (R80 and A05) and nontransgenic plants with or without stress. Trehalose accumulation under nonstressed (white bars), salt-stressed (100 mM NaCl for 4 weeks, hatched bars), or drought-stressed (100 h, black bars) conditions.

[0018] Figure 5 shows photosystem II electron transport rate in nontransformed and two independent, fifth generation transgenic plants grown under control conditions. The electron transport rate under increasing irradiance was calculated from chlorophyll fluorescence measurements on the youngest fully expanded leaf of NTC (▲), R80 (■), and A05 (●) at 360 ppm of CO_2, 25°C, and 50%

relative humidity after 10 weeks of growth. Values are the means ± SD (n = 9). Data are normalized to the average light-saturated rate of the nontransgenic control plants.

[0019] Figures 6 *A* and *B* show high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis of trehalose accumulation in a transgenic rice line. In Figure 6 *A*, the chromatogram shows the PAD-response profile from a leaf tissue extract of transgenic line A05. In Figure 6 *B*, the chromatogram shows the PAD-response profile of the same sample after digestion with trehalase enzyme. Arrow indicates the trehalose peak.

[0020] Figures 7 *A* and *B* show changes in the activity of photosystem II (ϕ_{PSII}) and ratio of variable to maximum fluorescence yields (Fv/Fm) during low-temperature stress, respectively. Five-week-old nontransformed and T_4 generation transgenic lines (R22, R38, R80 A05, A07, and A27) seedlings were exposed to 10° C for 72 h under a 10-h light/14-h dark photoperiod (photon flux density of 280 μmol photons m^{-2} s^{-1}) and a relative humidity of 50–60% and then allowed to recover under normal growth conditions at 25 ± 3°C for 24 h. Activity of ϕ_{PSII} and Fv/Fm were monitored for different time intervals during and after the low-temperature stress. Data represent means ± SD (n = 5) from independent plants. Figure 7 *A* shows Fv/Fm. Figure 7 *B* shows ϕ_{PSII}.

DETAILED DESCRIPTION OF THE INVENTION

[0021] The present invention relates to a transgenic monocot plant transformed with a nucleic acid encoding an enzyme for trehalose biosynthesis, under the control of an inducible promoter, that confers low temperature, salt, and water stress tolerance to a monocot plant.

[0022] The invention provides a method of producing a monocot plant cell or protoplast useful for regeneration of a low temperature stress, salt stress or water stress tolerant monocot plant by transforming a monocot plant cell or protoplast with a nucleic acid encoding an enzyme for trehalose biosynthesis under the control of an inducible promoter. Once transformation has occurred, the monocot plant cell or protoplast can be regenerated to form a transgenic monocot plant.

[0023] The present invention also relates to a method of conferring low temperature, salt, and water stress tolerance to a monocot plant by transforming a monocot plant cell or protoplast with a nucleic acid encoding an enzyme for trehalose biosynthesis, under control of an inducible promoter, under conditions effective to impart low temperature, salt, and water stress tolerance to monocot plants produced from the monocot plant cell or protoplast. This method includes transforming the monocot plant with an expression cassette comprising an inducible promoter and a nucleic acid encoding an enzyme for trehalose biosynthesis that confers low temperature, salt, and water stress tolerance to monocot plants, wherein the inducible promoter and the nucleic acid are operably linked together to permit expression of the nucleic acid. In a preferred embodiment, the inducible promoter is comprised of at least one ABRC unit and a minimal promoter. In another preferred embodiment, the at least one inducible element is a light-inducible rbcS promoter fragment with a chloroplast-targeting transit peptide.

[0024] Another aspect of the present invention further relates to a method of increasing tolerance of monocot plant to low temperature, salt, or water stress conditions by increasing the levels of an enzyme for trehalose biosynthesis in the monocot plant.

[0025] The present invention also relates to a transgenic monocot plant transformed with a plasmid that confers low temperature, salt, and water stress tolerance to the monocot plant where the plasmid comprises a first nucleic acid encoding trehalose-6-phosphate synthase, a first inducible promoter, the promoter located 5' to the first nucleic acid and controlling expression of the first nucleic acid, and a first termination sequence located 3' to the first nucleic acid.

[0026] Monocot plants, which can be transformed in accordance with the subject invention, are members of the family *Gramineae* (also known as *Poaceae*), and include rice (genus *Oryza*), wheat, maize (corn), barley, oat, rye, millet, and sorghum. Preferably, the cereal is rice, wheat, or corn, and most preferably the cereal is rice. Many species of cereals can be transformed, and, within each species, there are numerous subspecies and varieties that can be transformed. For example, within the rice species is subspecies Indica rice (Oryza sativa ssp. Indica), which includes the varieties IR36, IR64, IR72, Pokkali, Nona Bokra, KDML105, Suponburi 60,

Suponburi 90, Basmati 385, and Pusa Basmati 1. Another rice subspecies is Japonica, which includes Nipponbare, Kenfeng, and Tainung 67. Examples of suitable maize varieties include A188, B73, VA22, L6, L9, K1, 509, 5922, 482, HNP, and IGES. Examples of suitable wheat varieties include Pavon, Bob White, Hi-Line, Anza, Chris, Coker 983, FLA301, FLA302, Fremont, and Hunter.

[0027] Having identified the plant of interest, plant cells suitable for transformation include mature embryos, immature embryos, calli, suspension cells, and protoplasts. It is particularly preferred to use mature embryos and immature embryos.

[0028] In a preferred embodiment, the at least one ABRC unit is from a barley *HVA22* gene or a barley *HVA1* gene. The sequence for the at least one ABRC unit from a barley *HVA22* gene, a 49-bp ABA-responsive complex, is set forth in Shen et al., "Functional Dissection of an Abscisic Acid (ABA)-Inducible Gene Reveals Two Independent ABA-Responsive Complexes Each Containing a G-Box and Novel Acting Element," *The Plant Cell*, 7:295-307 (1995), which is hereby incorporated by reference in its entirety. The sequence for the ABRC unit from a barley *HVA1* gene is set forth in Shen et al., "Modular Nature of Abscisic Acid (ABA) Response Complexes: Composite Promoter Units that are Necessary and Sufficient for Induction of Gene Expression in Barley," *The Plant Cell*, 8:1107-1119 (1996). In a most preferred embodiment, up to four of the ABRC units are operably linked together in the expression cassette.

[0029] Suitable nucleic acids that increase tolerance to low temperature stress, salt stress, and water stress in monocot plants are genes the regulate the expression of stress-responsive genes and genes that encode enzymes involved in trehalose biosynthesis. Enzymes that encode trehalose biosynthesis can be isolated from a large number of organisms including bacteria, yeast, and invertebrates (see generally, Crowe et al., "Anhydrobiosis," *Annu. Rev. Physiol.*, 54:579-599 (1992), which is hereby incorporated by reference in its entirety). In a preferred embodiment, a nucleic acid that encodes an enzyme involved in trehalose biosynthesis is a DNA encoding trehalose-6-phosphate synthase. Preferably, the *TPS1* gene from yeast encodes the trehalose-6-phosphate synthase (for comparison of different yeast *TPS1* genes, see Kwon et al., "Cloning and Characterization of Genes Encoding Trehalose-6-

phosphate Synthase (TPS1) and Trehalose-6-phosphate Phosphatase (TPS2) from *Zygosaccharomyces rouxii*," *FEMS Yeast Res.*, 3:433-440 (2003), which is hereby incorporated by reference in its entirety). More preferably, the *otsA* gene from *Escherichia coli* encodes the trehalose-6-phosphate synthase. In another preferred embodiment, a nucleic acid that encodes an enzyme involved in trehalose biosynthesis is a DNA encoding trehalose-6-phosphate phosphatase. Preferably, the *TPS2* gene from yeast encodes the trehalose-6-phosphate phosphatase (for comparison of different yeast *TPS2* genes, see Kwon et al., "Cloning and Characterization of Genes Encoding Trehalose-6-phosphate Synthase (TPS1) and Trehalose-6-phosphate Phosphatase (TPS2) from *Zygosaccharomyces rouxii*," *FEMS Yeast Res.*, 3:433-440 (2003), which is hereby incorporated by reference in its entirety). More preferably, the *otsB* gene from *Escherichia coli* encodes the trehalose-6-phosphate phosphatase. In a more preferred embodiment, both the trehalose-6-phosphate synthase (*otsA*) and trehalose-6-phosphate phosphatase (*otsB*) are coexpressed in the monocot plant. In a most preferred embodiment, the trehalose-6-phosphate synthase (*otsA*) and trehalose-6-phosphate phosphatase (*otsB*) are expressed as a fusion protein in the moncot plant. The sequence of the *otsA* and *otsB* genes can be found in Kaasen et al., "Analysis of the *otsBA* Operon for Osmoregulatory Trehalose Synthesis in *Escherichia coli* and Homology of the OtsA and OtsB Proteins to the Yeast Trehalose-6-phosphate synthase/phosphatase complex," *Gene*, 145:9-15 (1994), which is hereby incorporated by reference in its entirety.

[0030] Suitable minimal promoters include Act1 of rice, rbcS of rice, a shortened α-amylase promoter of barley or rice, a shortened maize ubiquitin promoter, or a shortened CaMV 35S promoter.

[0031] In a preferred embodiment, the minimal promoter is an inducible promoter.

[0032] In a more preferred embodiment, the minimal promoter is the light inducible promoter rbcS of rice.

[0033] Most preferably, the minimal promoter is the stress inducible minimal Act1 promoter of rice and the sequence can be found in Su et al, "Dehydration Stress-regulate Transgene Expression in Stably Transformed Rice Plants," *Plant Physiol.*, 117:913-922 (1998), which is hereby incorporated by reference in its entirety.

[0034] In a preferred embodiment, the expression cassette comprising the inducible promoter and the nucleic acid encoding an enzyme for trehalose biosynthesis increases tolerance to low temperature stress, salt stress, and water stress in monocot plants.

[0035] These moncot plant cells are transformed with a nucleic acid, which could be RNA or DNA and which is preferably cDNA, encoding a molecule that increases tolerance to low temperature stress, salt stress, and water stress in monocot plants. The nucleic acid can be biologically isolated or synthetic and encodes for an enzyme for trehalose biosynthesis. In the following Examples, a key enzyme for biosynthesis, trehalose-6-phosphate synthase (TPS), is encoded by the *otsA* gene of *E. coli*. In the following Examples, a second key enzyme for biosynthesis, trehalose-6-phosphate phosphatase (TPP), is encoded by the *otsB* gene of *E. coli*.

[0036] Transformation of plant cells can be accomplished by using a plasmid. The plasmid is used to introduce the nucleic acid that increases tolerance to salt stress and drought stress in plants into the plant cell. Accordingly, a plasmid preferably includes a DNA molecule that increases tolerance to salt stress and drought stress in plants inserted into a unique restriction endonuclease cleavage site. Heterologous DNA, as used herein, refers to DNA not normally present in the particular host cell transformed by the plasmid. DNA is inserted into the vector using standard cloning procedures readily known in the art. This generally involves the use of restriction enzymes and DNA ligases, as described by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, which is hereby incorporated by reference in its entirety. The resulting plasmid, which includes a nucleic acid that, increases tolerance to salt stress and drought stress in plants can then be used to transform a host cell, such as an *Agrobacterium* and/or a plant cell. (See generally, *Plant Molecular Biology Manual*, 2d Edition, Gelvin et al., Eds., Kluwer Academic Press, Dordrecht, Netherlands (1994), which is hereby incorporated by reference in its entirety).

[0037] For plant transformation, the plasmid preferably also includes a selectable marker for plant transformation. Commonly used plant selectable markers include the hygromycin phosphotransferase *(hpt)* gene, the phosphinothricin acetyl transferase gene *(bar)*, the 5-enolpyruvylshikimate-3-phosphatesynthase gene

(EPSPS), neomycin 3'-0-phosphotransferase gene (*npt* II), or acetolactate synthase gene (ALS). Information on these selectable markers can be found in "Markers for Plant Gene Transfer" in *Transgenic Plants*, Kung et al., Eds., Vol. 1, pp. 89-123, Academic Press, NY (1993), which is hereby incorporated by reference in its entirety. In a preferred embodiment, the plasmid includes the phosphinothricin acetyl transferase gene *(bar)* in a selection cassette as a selectable marker for plant transformation under control of the cauliflower mosaic virus 35S promoter.

[0038] In a preferred embodiment, the plasmid is designated pSB109-TPSP or pSB-RTSP, each of which includes an *otsA* and *otsB* fusion gene.

[0039] For plant transformation, the plasmid also preferably includes a nucleic acid molecule encoding a 3' terminator such as that from the 3' non-coding region of genes encoding a proteinase inhibitor, actin 1, or nopaline synthase (nos). In a preferred embodiment, the plasmid includes a nucleic acid molecule encoding the 3' non-coding region of the proteinase inhibitor II gene (pinII) as a 3' terminator for the expression cassette comprising the inducible promoter and the nucleic acid encoding an enzyme for trehalose biosynthesis. Preferably, the plasmid includes a nucleic acid molecule encoding 3' non-coding region of the nopaline synthase gene (nos) as a 3' terminator for the selection cassette for plant transformation.

[0040] Other suitable plasmids for use in the subject invention can be constructed. For example, genes encoding a nucleic acid that increases trehalose biosynthesis and that increases tolerance to low temperature stress, salt stress, and water stress in monocot plants other than the *otsA* gene or the *otsB* gene of *E. coli* could be ligated into the parent plasmid SB109-TPSP or SB-RTSP after use of restriction enzymes to remove the *otsA* gene, the *otsB* gene, or the *otsA/otsB* fusion gene. Other minimal promoters could replace the rice actin 1 gene promoter present in plasmid SB109-TPSP or the rbcS gene promoter in plasmid SB-RTSP. Alternatively, other plasmids in general containing genes encoding a nucleic acid that increases trehalose biosynthesis and that increases tolerance to low temperature stress, salt stress, and water stress in monocot plants under the control of a suitable minimal promoter, with suitable selectable markers, can be readily constructed using techniques well known in the art.

[0041] Having identified the plasmid, one technique of transforming monocot plant cells with a nucleic acid that increases tolerance to low temperature stress, salt stress, and water stress in plants is by contacting the plant cell with an inoculum of an *Agrobacterium* bacteria transformed with the plasmid comprising the nucleic acid that increases tolerance to low temperature stress, salt stress, and water stress in monocot plants. Generally, this procedure involves inoculating the plant cells with a suspension of the transformed bacteria and incubating the cells for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

[0042] Bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Suitable species include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *Agrobacterium tumefaciens* (e.g., strains LBA4404 or EHA105) is particularly useful due to its well-known ability to transform plants.

[0043] In inoculating the cells of plants with *Agrobacterium* according to the subject invention, the bacteria must be transformed with a vector, which includes a gene encoding for an enzyme for trehalose biosynthesis.

[0044] Plasmids, suitable for incorporation in *Agrobacterium*, which include a nucleic acid that increases tolerance to low temperature stress, salt stress, and water stress in plants, contain an origin of replication for replication in the bacterium *Escherichia coli*, an origin of replication for replication in the bacterium *Agrobacterium tumefaciens*, T-DNA right border sequences for transfer of genes to plants, and marker genes for selection of transformed plant cells. Particularly preferred is the vector pBI121, which contains a low-copy RK2 origin of replication, the neomycin phosphotransferase (nptII) marker gene with a nopaline synthase (NOS) promoter and a NOS 3' polyadenylation signal. T-DNA plasmid vector pBI121 is available from Clontech Laboratories, 4030 Fabian Way, Palo Alto, California 94303. A nucleic acid that increases tolerance to low temperature stress, salt stress, and water stress in monocot plants is inserted into the vector to replace the beta-glucuronidase (GUS) gene.

[0045] Typically, *Agrobacterium* spp. are transformed with a plasmid by direct uptake of plasmid DNA after chemical and heat treatment, as described by Holsters et al., "Transfection and Transformation of *Agrobacterium tumefaciens*," *Mol. Gen. Genet.*, 163:18 1-187 (1978), which is hereby incorporated by reference in its entirety;

by direct uptake of DNA after electroporation, as described by Shen et al., "Efficient Transformation of *Agrobacterium* spp. by High Voltage Electroporation," *Nucleic Acids Research*, 17: 8385 (1989), which is hereby incorporated by reference in its entirety; by triparental conjugational transfer of plasmids from *Escherichia coli* to *Agrobacterium* mediated by a Tra+ help strain as described by Ditta et al., "Broad Host Range DNA Cloning System for Gram-negative Bacteria: Construction of a Gene Bank of *Rhizobium meliloti*," *Proc. Natl. Acad. Sci. USA*, 77:7347-7351 (1981), which is hereby incorporated by reference in its entirety; or by direct conjugational transfer from *Escherichia coli* to *Agrobacterium* as described by Simon et al., "A Broad Host Range Mobilization System for *in vivo* Genetic Engineering: Transposon Mutagenesis in Gram-Negative Bacteria," *Biotechnology*, 1:784-791 (1982), which is hereby incorporated by reference in its entirety.

[0046] Another method for introduction of a containing nucleic acid encoding an enzyme for trehalose biosynthesis into a plant cell is by transformation of the plant cell nucleus, such as by particle bombardment. As used throughout this application, particle bombardment (also known as biolistic transformation) of the host cell can be accomplished in one of several-ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference in its entirety. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the plasmid can be introduced into the cell by coating the particles with the plasmid containing the heterologous DNA. Alternatively, the target cell can be surrounded by the plasmid so that the plasmid is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the plasmid and heterologous DNA) can also be propelled into plant cells.

[0047] A further method for introduction of the plasmid into a plant cell is by transformation of plant cell protoplasts (stable or transient). Plant protoplasts are enclosed only by a plasma membrane and will therefore take up macromolecules like heterologous DNA. These engineered protoplasts can be capable of regenerating whole plants. Suitable methods for introducing heterologous DNA into plant cell

protoplasts include electroporation and polyethylene glycol (PEG) transformation. As used throughout this application, electroporation is a transformation method in which, generally, a high concentration of DNA (containing heterologous DNA) is added to a suspension of host cell protoplasts and the mixture shocked with an electrical field of 200 to 600 V/cm. Following electroporation, transformed cells are identified by growth on appropriate medium containing a selective agent.

[0048] As used throughout this application, transformation encompasses stable transformation in which the plasmid is integrated into the plant chromosomes.

[0049] In the Examples which follow, rice has been transformed using the *Agrobacterium* method as described in Hiei et al., "Efficient Transformation of Rice (*Oryza sativa* L.) Mediated by *Agrobacterium* and Sequence Analysis of the Boundaries of the T-DNA," *The Plant Journal*, 6:271-282 (1994), which is hereby incorporated by reference in its entirety. biolistic transformation. Other methods of transformation have also been used to successfully transform rice plants, including the protoplast method (for a review, see Cao et al., "Regeneration of Herbicide Resistant Transgenic Rice Plants Following Microprojectile-Mediated Transformation of Suspension Culture Cells," *Plant Cell Rep.*, 11:586-591 (1992), which is hereby incorporated by reference in its entirety), and the biolistic transformation method (disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference in its entirety). Biolistic transformation has been used successfully to transform wheat (for a review, see Weeks et al., "Rapid Production of Multiple Independent Lines of Fertile Transgenic Wheat (*Triticum aestivum*)," *Plant Physiol.*, 102:1077-1084 (1993), which is hereby incorporated by reference in its entirety). Biolistic transformation has also been used to successfully transform maize (for a review, see Mackey et al., "Transgenic Maize," In *Transgenic Plants*, Kung et al., Eds., vol. 2, pp. 21-33 (1993), which is hereby incorporated by reference in its entirety) and wheat (see Patent No. 5,405,765 to Vasil et al., which is hereby incorporated by reference in its entirety).

[0050] Once a monocot plant cell or protoplast is transformed in accordance with the present invention, it is regenerated to form a transgenic monocot plant. Generally, regeneration is accomplished by culturing transformed cells or protoplasts on medium containing the appropriate growth regulators and nutrients to allow for the

initiation of shoot meristems. Appropriate antibiotics are added to the regeneration medium to inhibit the growth of *Agrobacterium* or other contaminants and to select for the development of transformed cells or protoplasts. Following shoot initiation, shoots are allowed to develop in tissue culture and are screened for marker gene activity.

[0051] In suitable transformation methods, the monocot plant cell to be transformed can be *in vitro* or *in vivo*, i.e. the monocot plant cell can be located in a monocot plant.

[0052] The present invention also relates to a transgenic monocot plant transformed with a nucleic acid that increases tolerance to low temperature stress, salt stress, and water stress operably linked to an inducible promoter.

[0053] The invention also provides seed produced by the transgenic monocot plant. The invention is also directed to seed, which upon germination, produces the transgenic monocot plant.

[0054] Also encompassed by the present invention are transgenic monocot plants transformed with fragments of the nucleic acids that increase tolerance to low temperature stress, salt stress, and water stress of the present invention. Suitable fragments capable of conferring low temperature stress, salt stress or water stress tolerance to monocot plants can be constructed by using appropriate restriction sites. A fragment refers to a continuous portion of the nucleic acid that increases tolerance to salt stress and drought stress that is less than the entire molecule.

[0055] Non-essential nucleotides could be placed at the 5' and/or 3' ends of the fragments (or the full length nucleic acids that increase tolerance to salt stress and drought stress) without affecting the functional properties of the fragment or molecule (i.e. in increasing water stress or salt stress tolerance). For example, the nucleic acid that increases tolerance to low temperature stress, salt stress, and water stress may be conjugated to a signal (or leader) sequence at the N-terminal end (for example) of the nucleic acid that increases tolerance to low temperature stress, salt stress, and water stress which co-translationally or post-translationally directs transfer of the nucleic acid that increases tolerance to low temperature stress, salt stress, and water stress. The nucleotide sequence may also be altered so that the nucleic acid that increases

tolerance to low temperature stress, salt stress, and water stress is conjugated to a linker or other sequence for ease of synthesis, purification, or identification.

[0056] The transgenic cereal plant cell or protoplast or plant can also be transformed with a nucleic acid encoding a selectable marker, such as the *bar* gene, to allow for detection of transformants, and with a nucleic acid encoding the cauliflower mosaic virus 35S promoter to control expression of the *bar* gene. Other selectable markers include genes encoding EPSPS, nptII, or ALS. Other promoters include those from genes encoding actin 1, rbcS, ubiquitin, and PINII. These additional nucleic acid sequences can also be provided by the plasmid encoding a gene that imparts tolerance to low temperature stress, salt stress, and water stress and its promoter. Where appropriate, the various nucleic acids could also be provided by transformation with multiple plasmids.

[0057] While the nucleic acid that increases tolerance to low temperature stress, salt stress, and water stress referred to herein encodes, for example, a gene that imparts tolerance to low temperature stress, salt stress, and water stress, nucleotide identity to previously sequenced to low temperature stress, salt stress, and water stress genes is not required. As should be readily apparent to those skilled in the art, various nucleotide substitutions are possible which are silent mutations (i.e. the amino acid encoded by the particular codon does not change). It is also possible to substitute a nucleotide which alters the amino acid encoded by a particular codon, where the amino acid substituted is a conservative substitution (i.e. amino acid "homology" is conserved). It is also possible to have minor nucleotide and/or amino acid additions, deletions, and/or substitutions in the low temperature stress, salt stress, and water stress gene nucleotide and/or amino acid sequences which have minimal influence on the properties, secondary structure, and hydrophilic/hydrophobic nature of the encoded low temperature stress, salt stress, and water stress gene. These variants are encompassed by the present invention.

EXAMPLES

Example 1 – Plasmid Construction for Rice Transformation

[0058] Two binary plasmids, pSB109-TPSP and pSB-RTSP, each containing a *TPSP* fusion gene (Seo et al., "Characterization of a Bifunctional Enzyme Fusion of Trehalose-6-Phosphate Synthetase and Trehalose-6-Phosphate Phosphatase of *Escherichia coli*," *Appl. Environ. Microbiol.*, 66:2484-2490 (2000), which is hereby incorporated by reference in its entirety), were constructed in the pSB11 vector (Komari et al., "Vectors Carrying Two Separate T-DNAs for Co-Transformation of Higher Plants Mediated by Agrobacterium tumefaciens and Segregation of Transformants Free from Selection Markers," *Plant J.*, 10:165-174 (1996), which is hereby incorporated by reference in its entirety) by using standard cloning and plasmid manipulation procedures. The components of the plasmid within the T-DNA region and the selected restriction enzyme sites are shown in Fig. 1 *A*, *B*, and *C*. The expression cassette in pSB109-TPSP consists of an abscisic acid (ABA)-inducible promoter (Su et al., "Dehydration-Stress-Regulated Transgene Expression in Stably Transformed Rice Plants," *Plant Physiol.*, 117:913- 922 (1998), which is hereby incorporated by reference in its entirety) that contains four tandem copies of ABA-inducible element ABRC1 (0.18 kb) coupled with a minimal rice actin 1 promoter (0.18 kb) and an HVA22 intron (0.24 kb). It is linked to the *TPSP* coding region (2.2 kb), which was constructed by fusing the *otsA* and *otsB* genes from *E. coli* after the stop codon of the *otsA* gene had been removed by PCR (Seo et al., "Characterization of a Bifunctional Enzyme Fusion of Trehalose-6-Phosphate Synthetase and Trehalose-6-Phosphate Phosphatase of *Escherichia coli*," *Appl. Environ. Microbiol.*, 66:2484-2490 (2000), which is hereby incorporated by reference in its entirety) and then ligated to the potato protease inhibitor II gene (*pinII*) 3' noncoding sequence (1.0 kb). The selection cassette includes the cauliflower mosaic virus 35S promoter (0.74 kb), phosphinothricin acetyltransferase gene (*bar*, 0.59 kb), and the nopaline synthase gene 3' noncoding sequence (Nos 3', 0.28 kb). In pSB-RTSP, a 1.3-kb fragment of the rice *rbcS* promoter (Kyozuka et al., "Light-Regulated and Cell-Specific Expression of Tomato rbcS-gusA and Rice rbcS-gusA Fusion

Genes in Transgenic Rice," *Plant Physiol.*, 102:991-1000 (1993), which is hereby incorporated by reference in its entirety) with a chloroplast-targeting transit peptide (0.16 kb) is linked to the *TPSP* coding region; the remaining components are similar to those in pSB109-TPSP. During the cloning and ligation of an ≈3.7-kb DNA fragment containing the rbcS promoter/transit peptide and *TPSP* fusion gene into the plasmid pSB-RTSP, three additional restriction sites (*Sac*I, *Sal*I, and *Hin*dIII) were added between *TPSP* and 3' *pin II*. Both the plasmids (pSB109-TPSP and pSB-RTSP) were separately transferred to *Agrobacterium tumefaciens* strain LBA4404 harboring the pSB1 vector (Komari et al., "Vectors Carrying Two Separate T-DNAs for Co-Transformation of Higher Plants Mediated by Agrobacterium tumefaciens and Segregation of Transformants Free from Selection Markers," *Plant J.*, 10:165-174 (1996), which is hereby incorporated by reference in its entirety) through triparental mating using the helper plasmid pRK2013. For cocultivation, the bacteria were grown from a single colony in liquid AB medium containing 50 mg/liter spectinomycin at 30°C for 3 days and were suspended at a density of 3 x 10^9 cells per ml in AAM medium (Hiei et al., "Efficient Transformation of Rice (*Oryza sativa* L.) Mediated by *Agrobacterium* and Sequence Analysis of the Boundaries of the T-DNA," *Plant J.*, 6:271-282 (1994), which is hereby incorporated by reference in its entirety) for rice transformation.

Example 2 – Production of Transgenic Rice Plants

[0059] Mature seeds of indica rice variety PB-1 were dehusked and sterilized in 70% (vol/vol) ethanol for 2-3 min and then transferred into 50% (vol/vol) Clorox solution for 40 min with gentle shaking. The seeds were rinsed several times with sterile water. The sterilized PB-1 seeds were then plated for callus induction on Murashige and Skoog (MS) medium (Sigma) supplemented with 3.0 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D)/0.2 mg/liter 6-benzylaminopurine (BAP)/300 mg/liter casein hydrolysate (CH)/30 g/liter maltose/3.0 g/liter phytagel, pH 5.8 (MSCl) and grown for 21 days at 25°C in the dark. Three weeks after callus induction from the scutellar region of the rice embryo, 150 embryogenic calli were immersed in *A. tumefaciens* suspension for 10 min. Infected calli were cocultivated in

MSCl medium supplemented with 10 g/liter glucose/100 μM acetosyringone, pH 5.2 (MSCC). After 3 days of cocultivation, calli were washed with sterile water containing 250 mg/liter cefotaxime and blotted on filter paper. The calli were immediately plated on a selection medium, MSCl medium, supplemented with 6 mg/liter bialaphos and 250 mg/liter cefotaxime, pH 5.8 (MSS), and incubated at 25°C in the dark for 2-3 weeks. The microcalli that had proliferated after the initial selection were further subcultured for two selection cycles on fresh MSS medium every 2 weeks. The actively dividing bialaphos-resistant calli were plated on MS plant regeneration medium containing 2.5 mg/liter BAP/1.0 mg/liter kinetin/0.5 mg/liter naphthaleneacetic acid (NAA)/300 mg/liter CH/30 g/liter maltose/4 mg/liter bialaphos/250 mg/liter cefotaxime/2.0 g/liter phytagel, pH 5.8 (MSPR) and grown at 25°C for a 10-h light/14-h dark photoperiod for 3-4 weeks. The regenerated plantlets were acclimatized hydroponically in Yoshida nutrient solution (Yoshida et al., *Laboratory Manual for Physiological Studies of Rice*, International Rice Research Institute, Manila, Philippines, pp. 61-66 (1976), which is hereby incorporated by reference in its entirety), for 10 days. Later on, putative primary transformants (T_0 generation) were transferred to pots and tested for Basta-herbicide resistance (Roy and Wu, "Arginine Decarboxylase Transgene Expression and Analysis of Environmental Stress Tolerance in Transgenic Rice," *Plant Sci.* 160:869-875 (2001), which is hereby incorporated by reference in its entirety); the transgenic plants were grown to maturity in a greenhouse for further analysis.

Example 3 – DNA Blot Hybridization Analysis of Transgenic Rice Plants

[0060] Leaves from nontransformed control (NTC) plant, and representative (T_0) transformants of nine A-lines (ABA-inducible promoter) and five R-lines (rbcS promoter) that were transformed with the plasmid pSB109-TPSP and pSB-RTSP, respectively, were ground in liquid nitrogen by using a mortar and pestle. Rice genomic DNA was isolated by the guanidine-detergent lysis method by using DNAzolES (Molecular Research Center, Cincinnati) following the manufacturer's instructions. Five micrograms of the genomic DNA was digested overnight with *Hin*dIII restriction enzyme, fractionated through 0.8% agarose gel, alkali-transferred

onto Hybond *N*+ nylon membrane (Amersham Pharmacia), and hybridized with an α-^{32}P-labeled 2.2-kb *TPSP* fusion gene (Seo et al., "Characterization of a Bifunctional Enzyme Fusion of Trehalose-6-Phosphate Synthetase and Trehalose-6-Phosphate Phosphatase of *Escherichia coli*," *Appl. Environ. Microbiol.*, 66:2484-2490 (2000), which is hereby incorporated by reference in its entirety) as the probe. DNA probe preparation, hybridization, and washing of the membrane were performed as described (Roy and Wu, "Arginine Decarboxylase Transgene Expression and Analysis of Environmental Stress Tolerance in Transgenic Rice," *Plant Sci.* 160:869-875 (2001), which is hereby incorporated by reference in its entirety). The α-^{32}P-labeled membrane was exposed onto autoradiogram.

Example 4 – Detecting Trehalose and Soluble Carbohydrates

[0061] Soluble carbohydrates were extracted as described (Goddijn et al., "Inhibition of Trehalase Activity Enhances Trehalose Accumulation in Transgenic Plants," *Plant Physiol.*, 113:181-190 (1997), which is hereby incorporated by reference in its entirety). Extracts from 0.5 g of homogenized fresh leaf tissue were centrifuged (10 min at 3,220 x *g*); supernatants were passed through ion-exchange columns consisting of 1 ml of Amberlite IR-68 (acetate form) layered on 1 ml of Dowex 50W (hydrogen form) to remove charged compounds. After lyophilization, samples were dissolved in HPLC-grade water and subjected to high-performance anion exchange chromatography with pulsed amperometric detection by using a Dionex DX-500 series chromatograph equipped with a Carbopac PA-1 analytical column and a Carbopac PA-1 guard column (Dionex). Carbohydrates were eluted at a flow rate of 1.0 ml per min at 1,400 psi with 100 mM NaOH for 34 min. Major soluble carbohydrates present were quantified by using authentic standard sugars (Sigma). The identity of trehalose in the plant extracts was confirmed by incubating samples with porcine-kidney-derived trehalase enzyme (Sigma).

Example 5 – Determination of Salt Stress Tolerance and Plant Mineral Nutrients

[0062] Ten seedlings for each T_4 generation transgenic line (R22, R38, R80, A05, A07, and A27) and NTC were grown hydroponically (with modest aeration) in

Yoshida nutrient solution (Yoshida et al., *Laboratory Manual for Physiological Studies of Rice*, International Rice Research Institute, Manila, Philippines, pp. 61-66 (1976), which is hereby incorporated by reference in its entirety) in a growth chamber at 25 ± 3°C for a 10-h light/14-h dark photoperiod (photon flux density of 280 µmol photons per m/s) and with relative humidity of 50-60%. After 5 weeks, 50% of the seedlings were subjected to 100 mM NaCl stress (conductivity of 10-12 dS/m). Nutrient solutions were replaced every week. After 4 weeks of continuous salt stress, shoot and root samples were separately harvested for fresh and dry weight determination. For mineral nutrient analysis, 150 mg of ground dry matter was digested in concentrated HNO_3 overnight at 120°C. Samples then were dissolved in HNO_3:$HClO_4$ (1:1, vol/vol) at 220°C, resuspended in 5% (vol/vol) HNO_3, and analyzed for elemental composition of sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and iron (Fe) by means of simultaneous inductively coupled argon-plasma emission spectrometry (ICP trace analyzer; Plant, Soil, and Nutrition Laboratory, U.S. Department of Agriculture-Agriculture Research Service, Cornell University, Ithaca, NY).

Example 6 – Determination of Drought and Low-Temperature Stress Tolerance

[0063] Seedlings from six independent T_4 transgenic lines and nontransformed line were grown individually in 10-cm x 10-cm pots irrigated with Yoshida nutrient solution for 5 weeks before performing the drought- or low-temperature stress experiment. Drought stress (water deficit) was conducted by first withholding irrigation for 3 days to allow the soil in the pot to dry. Then, the first drought cycle of 100 h was initiated, followed by rewatering for 2 days. The drought-stress cycle was repeated for another 100 h, and the plants were allowed to recover by watering every day for 3 weeks. Low-temperature stress was conducted on five-week-old seedlings by exposing them to 10°C for 72 h under a 10-h light/14-h dark photoperiod (photon flux density of 280 µmol photons per m per s) and a relative humidity of 50-60%; the seedlings were then allowed to recover under normal growth conditions at 25 ± 3°C.

Example 7 – Protein Extraction and Immunoblotting

[0064] Proteins were extracted from 0.2 g of homogenized fresh leaf tissue in protein extraction buffer (20 mM Tris·HCl, pH 8.0/10 mM EDTA/30 mM NaCl/2 mM phenylmethane sulfonyl fluoride for 1 h at 4°C). The homogenate was clarified by centrifugation at 12,000 x *g* for 15 min at 4°C. The procedure for immunoblotting was essentially the same as described (Xu et al., "Expression of a Late Embryogenesis Abundant Protein Gene, HVA1, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice," *Plant Physiol.* 110:249-257 (1996), which is hereby incorporated by reference in its entirety). The anti-TPSP protein polyclonal antibody was used at a 1:1,500 dilution for Western blot analysis, using an alkaline phosphatase color reaction for detection of the protein, as per the manufacturer's instruction (Bio-Rad).

Example 8 – Chlorophyll Fluorescence Parameters

[0065] Fv/Fm and ϕ_{PSII} were measured by using a pulse amplitude modulated fluorometer (FMS2, Hansatech Instruments, Pentney King's Lynn, U.K.) to estimate photo-oxidative damage to the Photosystem II (PS II) reaction center and the quantum efficiency of PS II photochemistry under ambient light conditions, respectively, as described (Saijo et al., "Over-Expression of a Single Ca2+-Dependent Protein Kinase Confers Both Cold and Salt/Drought Tolerance on Rice Plants," *Plant J.* 23:319-327 (2000), which is hereby incorporated by reference in its entirety). Measurements were made on the youngest, fully expanded leaves. Measurements of ϕ_{PSII} were first determined under ambient light; the same leaves were then dark-adapted for 10 min before measurement of Fv/Fm.

Example 9 – Transgenic Rice Plants with Enhanced Trehalose Levels Are Phenotypically Normal and Fertile

[0066] Two plasmid constructs, pSB109-TPSP (Fig. 1 *A*, *C*) and pSB-RTSP (Fig. 1 *B*, *C*), each containing the *TPSP* fusion gene, were introduced into *indica* rice cells of PB-1 by *Agrobacterium*-mediated gene transfer (Hiei et al., "Efficient Transformation of Rice (*Oryza sativa* L.) Mediated by *Agrobacterium* and Sequence

Analysis of the Boundaries of the T-DNA," *Plant J.*, 6:271-282 (1994), which is hereby incorporated by reference in its entirety). In the plasmid construct pSB109-TPSP, an ABA and stress-inducible promoter (Su et al., "Dehydration-Stress-Regulated Transgene Expression in Stably Transformed Rice Plants," *Plant Physiol.*, 117:913- 922 (1998), which is hereby incorporated by reference in its entirety) drives the fusion gene for cytosolic expression. In the other plasmid, pSB-RTSP, the light-regulated promoter (Kyozuka et al., "Light-Regulated and Cell-Specific Expression of Tomato rbcS-gusA and Rice rbcS-gusA Fusion Genes in Transgenic Rice," *Plant Physiol.*, 102:991-1000 (1993), which is hereby incorporated by reference in its entirety) of the Rubisco small subunit gene, *rbcS*, from *Oryza sativa* with a transit peptide drives the fusion gene for chloroplast targeting in the leaf mesophyll cells. A large number of putative transgenic PB-1 plants (T_0 generation) were regenerated (Table 1); these plants included 28 A-lines (ABA-inducible promoter) and 76 R-lines (rbcS promoter).

Table 1. Efficiency of rice transformation using *Agrobacterium tumefaciens* strain LBA 4404 (pSB1) containing the *TPSP* fusion gene in plasmids pSB109-TPSP and pSB-RTSP

Plasmid	pSB109-TPSP	pSB-RTSP
Promoter	ABA and stress-inducible	Rice rbcS with transit peptide
Expression Target	Cytosolic	Chloroplast
No. of calli co-cultivated	150	150
No. of bialophos resistant calli	41/150	118/150
No. of plants regenerated	29/41	89/118
No. of basta resistant plants	28/29	76/89
No. of fertile T_0 lines	22 (79 %)	68 (90 %)

Numbers in parenthesis indicate percentage of completely fertile plants.

Integration of the *TPSP* transgene was confirmed by DNA-blot hybridization analysis (Fig. 1 *D* and *E*). Based on the T-DNA junction fragment analysis, ≈40% of the

transgenic plants transformed with either of the plasmids harbor a single copy, and 35-45% of plants harbor two or three copies of the transgene.

[0067] Most of the 90 independent primary transformants (T_0) that contained a low copy number of the transgene showed a normal phenotype and were completely fertile. In contrast to previous reports that used constitutive promoters driving individual TPS and/or TPP genes, the use of stress-inducible or tissue-specific promoters in this work appears to minimize the negative effects of the transgene on plant growth. The T_0 plants were self-pollinated to obtain segregating T_1 progeny for genetic and HPLC analysis. Forty-five transgenic lines showed a segregation pattern of 3:1 for the basta-herbicide resistance marker gene. HPLC analysis of leaf extracts showed that transgenic lines had a trehalose content that was between three times and eight times that of the nontransgenic plants (17 ± 5 μg of trehalose per g of fresh weight). The identity of trehalose in the plant tissue extracts was confirmed by incubating samples in porcine trehalase followed by chromatographic analysis of the monosaccharide products (Figure 6). Physiological experiments were conducted for abiotic stress tolerance on homozygous plants through the T_4 generation, because gene silencing has been reported to occur in the T_3 generation, even though T_2 and T_1 generation plants were not silenced (Iyer et al., "Transgene Silencing in Monocots," *Plant Mol. Biol.*, 43:323-346 (2000), which is hereby incorporated by reference in its entirety). The results from many independent transgenic lines were consistent for salt- and drought-stress tolerance in each generation, except in few transgenic lines which had multiple copies of the transgene.

Example 10 – Transgenic Rice Plants Are Salt Tolerant and Maintain Balanced Mineral Nutrition

[0068] The T_4 transgenic plants with either one or two copies of the transgene showed markedly enhanced salt tolerance during and subsequent to 4 weeks of 100 mM NaCl treatment under hydroponic growth conditions. Six independent transgenic plant lines (three A-lines and three R-lines) were analyzed in detail. For clarity of presentation, results from two representative transgenic lines (R80 and A05) are shown (Figure 2); results for the other four lines were very similar to the two lines presented. After prolonged exposure to salt stress, almost all of the transgenic plants

survived and displayed vigorous root and shoot growth. In contrast, all of the nontransformed stressed (NTS) plants were either dead or nearly dead because of severe salt damage to the leaves and concomitant loss of chlorophyll. Transgenic plants developed longer and thicker roots than NTS plants after salt stress (Figure 2 *A*). Salt stress severely inhibited the growth of shoot and roots of NTS plants, as indicated by their lower dry weights compared with NTC plants. Shoot and root dry weights of both the transgenic lines (Figure 2 *B*) approached those of NTC plants, and, after removal of salt stress, the transgenic plants were able to grow, flower, and set normal viable seeds. To determine whether the *TPSP* gene product was present in the salt-stressed plants, total protein was isolated from the leaf samples for Western blot analysis. Immunoblot analysis using polyclonal antibodies raised against the fusion protein showed the presence of a protein with the expected apparent molecular mass of 88 kDa only in the transgenic plants (Figure 2 *C*).

[0069] To assess how trehalose accumulation in transgenic rice affected plant mineral nutrition during salt stress, shoot and root mineral content for the six independent transgenic lines and two nontransgenic lines were determined by using inductively coupled plasma emission spectrometry (Table 2).

Table 2. Plant mineral nutrient content (sodium, potassium, calcium, and iron ions) in shoots and roots of transgenic lines (R22, R38, R80 A05, A07, and A27) and nontransformed control lines with or without salt stress

Line	Na		K		Ca		Fe	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Nonstress conditions								
NTC-1	1.4 ± 0.3	1.2 ± 0.1	33 ± 8	18 ± 6	2.5 ± 0.6	0.6 ± 0.1	0.13 ± 0.08	2.8 ± 1.5
NTC-2	1.1 ± 0.3	1.3 ± 0.3	33 ± 3	15 ± 2	2.3 ± 0.1	0.7± 0.1	0.13 ± 0.04	3.5 ± 0.7
R22	1.2 ± 0.3	1.1 ± 0.4	30 ± 2	21 ± 1	2.5 ± 0.5	1.6 ± 0.2	0.20 ± 0.09	6.1 ± 3.0
R38	0.9 ±	1.8 ±	35 ± 5	23 ± 4	2.3 ±	1.0 ±	0.16 ±	3.7 ±

	0.2	0.2			0.5	0.3	0.03	0.4
R80	1.2 ± 0.2	1.5 ± 0.2	44 ± 1	23 ± 1	2.8 ± 0.7	0.5 ± 0.1	0.28 ± 0.01	4.3 ± 1.1
A05	1.0 ± 0.3	1.5 ± 0.8	45 ± 1	22 ± 4	2.7 ± 0.5	0.6 ± 0.1	0.21 ± 0.04	4.3 ± 0.4
A07	2.2 ± 0.7	1.1 ± 0.2	45 ± 1	24 ± 1	3.7 ± 1.2	0.9 ± 0.2	0.25 ± 0.07	4.3 ± 0.2
A27	1.9 ± 0.4	1.0 ± 0.3	47 ± 4	20 ± 1	2.4 ± 0.8	1.5 ± 0.2	0.19 ± 0.01	5.1 ± 2.9

Salt-stress conditions (100 mM NaCl)								
NTS-1	77 ± 13	7 ± 3	31 ± 4	6 ± 2	4.4 ± 0.8	1.8 ± 0.3	0.25 ± 0.08	9.3 ± 2.7
NTS-2	87 ± 17	4 ± 1	30 ± 2	3 ± 1	5.6 ± 0.5	1.7 ± 0.3	0.24 ± 0.05	8.3 ± 3.4
R22	34 ± 10	6 ± 2	26 ± 2	16 ± 2	4.6 ± 0.7	1.0 ± 0.3	0.47 ± 0.11	5.8 ± 2.3
R38	24 ± 10	7 ± 1	30 ± 2	17 ± 4	4.1 ± 0.8	0.5 ± 0.1	0.42 ± 0.07	4.7 ± 1.5
R80	28 ± 7	6 ± 2	29 ± 2	17 ± 2	4.2 ± 0.6	0.7 ± 0.1	0.47 ± 0.08	6.8 ± 2.6
A05	30 ± 10	6 ± 1	34 ± 5	17 ± 1	4.2 ± 0.8	0.5 ± 0.2	0.46 ± 0.03	7.8 ± 1.4
A07	24 ± 8	7 ± 1	29 ± 2	18 ± 3	2.7 ± 0.5	0.5 ± 0.1	0.48 ± 0.04	5.3 ± 0.8
A27	18 ± 7	7 ± 3	29 ± 4	20 ± 1	3.0 ± 0.5	0.5 ± 0.3	0.45 ± 0.05	5.5 ± 1.4

The ionic concentration is presented as mg/g shoot or roots dry weight. Values are the means ± SD (n = 5).

After continuous salt stress (100 mM NaCl) for 4 weeks, NTS plants showed a very large increase in Na^+ content in both shoots and roots compared with NTC, whereas the increase in the shoots of all of the transgenic plants was much smaller (Figure 2 *D*). The Na^+ content of transgenic plant shoots was only 30-35% of the NTS plants after salt stress. The observed differences in shoot Na^+ content between transgenic and NTS plants could be caused in part by a growth dilution because of the much faster growth rate of the transgenic plants under salt stress. Alternatively, trehalose might have played a direct or indirect role in maintaining ion selectivity and, thus, facilitating cellular Na^+ exclusion. This possibility is consistent with the report that in salt-stressed rice seedlings, the accumulation of Na^+ in leaf tissues was not prevented by exogenous proline. In contrast, treatment with exogenous trehalose significantly reduced the salt-induced accumulation of Na^+ in the leaves (Garcia et al., "Effects of

Osmoprotectants Upon NaCl Stress in Rice," *Plant Physiol.*, 115:159-169 (1997), which is hereby incorporated by reference in its entirety).

[0070] Transgenic lines R80 and A05 maintained shoot to root K^+ homeostasis both under nonstress and salt-stress conditions (Table 2). After salt stress, the levels of shoot and root K^+ content in transgenic plants was similar to the nonstressed controls, while a fourfold decrease in root K^+ in the NTS plants was seen (Figure 2 *E*). Thus, the transgenic plants were able to maintain a higher level of selectivity for K^+ over Na^+ uptake in the roots and Na^+ exclusion from the shoots compared with the NTS plants. The maintenance of the Na^+/K^+ ratio in both shoot and roots of transgenic plants (Figure 2 *F*) correlated with nearly normal plant growth and may be the basis for minimizing Na^+ toxicity under salt stress. It is generally accepted that the maintenance of Na^+/K^+ homeostasis is an important aspect of salt tolerance (Rus et al., "AtHKT1 is a Salt Tolerance Determinant that Controls Na^+ Entry into Plant Roots," *Proc. Natl. Acad. Sci. USA*, 98:14150-14155 (2001) and Epstein, "Plant Biology: How Calcium Enhances Plant Salt Tolerance," *Science*, 280:1906-1907 (1998), which are hereby incorporated by reference in their entirety).

[0071] Several other changes in plant mineral status that may have played indirect roles in stress tolerance were seen in the transgenic lines compared with the NTCs. It was found that salt stress led to a significant increase in root and shoot Ca^{2+} content in the NTS lines, whereas in the transgenic lines, this Na-mediated increase in Ca^{2+} content was only observed in the shoots and not the roots (Table 2). This rise in Ca^{2+} may be caused by alterations in the ion selectivity of the transporters at high concentrations of Na^+ (Epstein, "Plant Biology: How Calcium Enhances Plant Salt Tolerance," *Science*, 280:1906-1907 (1998), which is hereby incorporated by reference in its entirety). Significantly higher levels of shoot Fe ion content were also found in the transgenic lines compared with the NTCs (Table 2). It has been well documented that Fe, Cu, and Zn ions are essential for the function of critical antioxidant enzymes such as the superoxide dismutases that play a role in scavenging reactive oxygen species during a number of abiotic stresses (Epstein, "Plant Biology: How Calcium Enhances Plant Salt Tolerance," *Science*, 280:1906-1907 (1998), Alscher et al., "Role of Superoxide Dismutases (SODs) in Controlling Oxidative Stress in Plants," *J. Exp. Bot.*, 53:1331-1341 (2002), which are hereby incorporated

by reference in their entirety). In general, the relationship between salt stress and plant mineral content is complex, and the links between elevated trehalose content and improved mineral status during salt stress are not known.

Example 11 – Transgenic Rice Plants are Water Stress Tolerant

[0072] To study drought tolerance, 5-week-old nontransformed and transgenic seedlings grown in soil were subjected to two cycles of 100 h of drought stress. After the drought treatments, all 15 plants of each line showed wilting and drought-induced rolling of the young leaves. Nontransgenic plants exhibited rolling of leaves within 48 h of the stress as compared with considerably fewer visual symptoms in transgenic plants during the same time period. After two cycles of 100 h of drought stress and subsequent watering for 3 weeks, the growth of both the transgenic lines, R80 and A05 (Figure 3 *B*), were almost identical to nonstressed control plant (Figure 3 *A*). In contrast, the growth of the drought-stressed NTS was severely inhibited (Figure 3 *B*).

Example 12 – Transgenic Rice Plants Produced Increased Amounts of Trehalose and Other Soluble Carbohydrates

[0073] To evaluate whether trehalose accumulation in plants might act as a positive regulator of stress tolerance, the levels of trehalose and other soluble carbohydrates were measured (Table 3).

Table 3. Levels of trehalose, glucose, fructose, sucrose, and total soluble carbohydrate content in shoots of nontransformed (NT) and six transgenic rice lines (R22, R38, R80 A05, A07, and A27) grown under no stress, salt-stressed (100 mM NaCl for 4 weeks), or drought-stressed (after first 100-hr drought stress cycle) conditions

Line	Trehalose	Glucose	Fructose	Sucrose	Total
Nonstress conditions					
NTC-1	17 ± 5	3.9 ± 0.17	3.4 ± 0.61	46 ± 3.7	53 ± 4.4
NTC-2	16 ± 6	3.8 ± 0.36	3.1 ± 0.70	45 ± 5.1	52 ± 5.6
R22	98 ± 14	5.0 ± 0.39	4.5 ± 0.74	51 ± 1.3	61 ± 2.2
R38	71 ± 11	4.9 ± 0.27	4.3 ± 0.65	49 ± 3.6	58 ± 4.1
R80	55 ± 8	5.5 ± 0.30	4.9 ± 0.79	54 ± 4.5	64 ± 6.0
A05	48 ± 7	5.6 ± 0.33	5.2 ± 0.85	53 ± 9.6	64 ± 10
A07	62 ± 8	5.6 ± 0.44	5.1 ± 0.89	60 ± 8.3	71 ± 9.9
A27	54 ± 9	5.2 ± 0.41	4.6 ± 0.69	52 ± 6.7	62 ± 7.8
Drought-stress conditions					
NTS-1	53 ± 11	4.7 ± 0.75	4.0 ± 0.86	45 ± 2.6	54 ± 3.8
NTS-2	47 ± 8	4.6 ± 0.47	3.5 ± 0.95	49 ± 3.6	57 ± 4.7
R22	156 ± 19	4.9 ± 0.49	3.7 ± 0.78	57 ± 2.3	65 ± 3.5
R38	257 ± 26	5.6 ± 0.66	4.8 ± 0.47	57 ± 6.6	68 ± 7.2
R80	163 ± 23	4.3 ± 1.24	3.1 ± 0.81	51 ± 4.2	59 ± 4.9
A05	508 ± 48	3.7 ± 0.51	2.3 ± 0.41	60 ± 5.6	67 ± 6.4
A07	474 ± 103	4.0 ± 0.83	2.6 ± 0.71	56 ± 8.1	63 ± 10
A27	401 ± 69	3.8 ± 0.42	2.8 ± 0.39	27 ± 2.4	34 ± 3.1
Salt-stress conditions					
NTS-1	29 ± 6	3.5 ± 0.08	3.0 ± 0.04	35 ± 2.7	42 ± 3.9
NTS-2	34 ± 6	3.1 ± 0.11	2.6 ± 0.03	37 ± 2.7	42 ± 4.0
R22	69 ± 8	4.2 ± 0.12	4.0 ± 0.08	36 ± 2.1	44 ± 2.7
R38	130 ± 19	5.1 ± 0.47	5.2 ± 0.12	38 ± 2.4	48 ± 3.1
R80	76 ± 12	4.2 ± 0.15	3.9 ± 0.10	42 ± 3.0	50 ± 3.8
A05	91 ± 13	4.0 ± 0.14	3.3 ± 0.10	44 ± 4.7	51 ± 5.7
A07	75 ± 8	3.0 ± 0.12	2.3 ± 0.09	34 ± 2.5	40 ± 3.1
A27	143 ± 18	2.8 ± 0.14	1.9 ± 0.10	35 ± 4.9	40 ± 5.8

Means ± SD (n = 3) are presented. Soluble carbohydrate content data is presented as mg/g shoot fresh weight, except in the case of trehalose, where it is presented as μg/g fresh weight.

A low but significant amount of trehalose was detected in the shoots (17 μg/g fresh weight) of NTC plants; these levels increased significantly under salt or drought stresses. The transgenic plants grown under control conditions exhibited trehalose levels comparable with the NTS plants (Figure 4). After salt stress, the transgenic lines (R80 and A05) showed 2.5-3 times higher shoot trehalose levels compared with NTS plants, whereas after drought stress, trehalose levels in the transgenic lines increased 3- to 9-fold (Figure 4). Despite the similarities in tolerance levels exhibited by transgenic plants engineered to increase trehalose synthesis in either the cytosol or chloroplast, R-lines showed considerable protection at much lower trehalose concentrations during drought stress (Table 3). In general, there was no obvious relationship between trehalose accumulation and stress tolerance among the transgenic lines evaluated. On the other hand, the difference in trehalose levels between the transgenic and nontransgenic lines clearly correlates with increased tolerance to abiotic stress.

Example 13 – Transgenic Rice Plants Show Improved Photosystem II Function

[0074] During many different abiotic stresses, a reduction in photosynthesis and the subsequent production of reactive oxygen species are thought to be a major contributor to decreased plant performance and photooxidative damage. The effects of increased trehalose accumulation on photosynthesis during drought stress were assessed by determination of the quantum yield of PS II photochemistry (ϕ_{PSII}) by using *in vivo* chlorophyll fluorescence techniques (Saijo et al., "Over-Expression of a Single Ca^{2+}-Dependent Protein Kinase Confers Both Cold and Salt/Drought Tolerance on Rice Plants," *Plant J.*, 23:319-327 (2000), which is hereby incorporated by reference in its entirety). ϕ_{PSII} is a measure of the photosynthetic performance of the plant under ambient light conditions. After the first cycle of 100 h of drought stress, the quantum yield of PS II photochemistry in NTS plants decreased by ≈68%, whereas the activity of the two best-performing transgenic lines (R80 and A05) only decreased by 29-37% compared with the nonstressed controls (Figure 3 *C*). Similarly, drought-induced decreases in the fluorescence parameter Fv/Fm, which is a measure of accumulated photo-oxidative damage to PS II, were considerably smaller in the

transgenic lines than in the NTS plants (Figure 3 *D*). In other independent experiments, similar results were obtained for both low-temperature stress (Figure 7) and salt stress, indicating the common role that maintenance of photosynthetic capacity plays in tolerance to these stresses.

Example 14 – Transgenic Rice Plants Have Increased Capacity Under Nonstress Conditions

[0075] Improved photosynthesis under abiotic stress conditions is known to limit photo-oxidative damage and permit continued growth (Owens, "Processing of Excitation Energy by Antenna Pigments," in *Photosynthesis and the Environment*, Baker, ed., Kluwer, Dordrecht, The Netherlands, pp. 1-23 (1996), which is hereby incorporated by reference in its entirety) and is clearly suggested by the data in Figure 3. Under the same conditions, transgenic plants exhibited soluble carbohydrate levels that were ≈20% higher than those of corresponding NTC plants, including subtle changes in levels of glucose, fructose, and sucrose (Table 3). Both of these results are consistent with the suggestion that trehalose may be involved in sugar sensing and modulating carbon metabolism (Goddijn et al., "Trehalose Metabolism in Plants," *Trends Plant Sci.*, 4:315-319 (1999), Thevelein and Hohmann, "Trehalose Synthase: Guard to the Gate of Glycolysis in Yeast?" *Trends Biochem. Sci.*, 20:3-10 (1995), which are hereby incorporated by reference in their entirety). The ability of trehalose to modulate photosynthetic capacity has been demonstrated recently (Paul et al., "Enhancing Photosynthesis with Sugar Signals," *Trends Plant Sci.*, 6:197-200 (2001), which is hereby incorporated by reference in its entirety) in transgenic tobacco plants expressing *E. coli* trehalose biosynthetic genes. Plants with enhanced TPS expression exhibited a higher photosynthesis per unit of leaf area than nontransgenic controls, whereas those over-expressing TPP showed diminished rates of photosynthesis. These data lead them to conclude that it is trehalose-6-P and not trehalose that is modulating photosynthetic capacity (Paul et al., "Enhancing Photosynthesis with Sugar Signals," *Trends Plant Sci.*, 6:197-200 (2001), which is hereby incorporated by reference in its entirety).

[0076] Figure 5 shows the light intensity dependence of PS II electron transport rates, as determined by ϕ_{PSII} measurements (Saijo et al., "Over-Expression of

a Single Ca2+-Dependent Protein Kinase Confers Both Cold and Salt/Drought Tolerance on Rice Plants," Plant J. 23:319-327 (2000), which is hereby incorporated by reference in its entirety) for nontransgenic rice and transgenic lines R80 and A05 measured under control (nonstress) conditions. Although the differences in photosynthesis are small at limiting light intensities, at light saturation, the rates of photosynthesis in the transgenic plants are 5-15% higher than in the NTCs. At light saturation, photosynthetic rate is limited by the capacity of the dark reactions, in particular, the Calvin cycle and triose phosphate utilization in the cytoplasm (Owens, "Processing of Excitation Energy by Antenna Pigments," in *Photosynthesis and the Environment*, Baker, ed., Kluwer, Dordrecht, The Netherlands, pp. 1-23 (1996), which is hereby incorporated by reference in its entirety). Together with the observed higher levels of soluble carbohydrate under both stress and nonstress conditions (Table 3), the elevated levels of light-saturated photosynthesis in the transgenic plants supports the suggestion that in plants, trehalose acts as a regulator of sugar sensing and, thus, the expression of genes associated with carbon metabolism (Paul et al., "Enhancing Photosynthesis with Sugar Signals," *Trends Plant Sci.*, 6:197-200 (2001), which is hereby incorporated by reference in its entirety). The presence of a higher capacity for photosynthesis before stress provides a larger sink for the products of photosynthesis during stress, thus limiting the extent of excess-light-induced photooxidative damage and accounting, in part, for the more vigorous growth of the transgenic lines during stress. Interestingly, the higher efficiency of trehalose synthesis by the TPSP fusion gene product (Seo et al., "Characterization of a Bifunctional Enzyme Fusion of Trehalose-6-Phosphate Synthetase and Trehalose-6-Phosphate Phosphatase of *Escherichia coli*," *Appl. Environ. Microbiol.*, 66:2484-2490 (2000), which is hereby incorporated by reference in its entirety) would suggest that trehalose, rather than trehalose-6-P is leading the enhanced capacity for photosynthesis.

Example 15 – Production of Transgenic Wheat Plants

[0077] Immature embryos were isolated from greenhouse-grown wheat (*Triticum aestivum* L.) cv. Bob White spring wheat variety and precultured for 1-4

days in the dark on modified MS medium before bombardment, as reported by Weeks et al., "Rapid Production of Multiple Independent Lines of Fertile Transgenic Wheat (*Triticum aestivum*), *Plant Physiol.* 102:1077-1084 (1993), which is hereby incorporated by reference in its entirety. Preparation of gold particles and coating with plasmid DNA was carried out based on the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Osmotic treatment of target tissue before and after bombardment was performed. Bombarded tissue was placed on the same culture medium supplemented with 5 mg l^{-1} bialaphos (a gift from Dr. H. Anzai, Meiji Seika Kaisha, Japan) for 4 weeks at 25°C in the dark. Bialaphos-resistant calli were transferred to regeneration medium (MS medium containing 2% sucrose, 0.15 mg l^{-1} thidiazuron and 1 mg l^{-1} bialaphos) for 2-3 weeks at 25°C under a 16 h photoperiod (66 μmol m-2 s^{-1}). After ≈2 weeks, regenerated shoots were transferred to Magenta® boxes (Sigma, St. Louis, MO, USA) containing rooting medium (half-strength MS medium and 2 mg l^{-1} bialaphos) for 2-4 weeks at 25°C under the above light conditions.

[0078] Plantlets were transferred from rooting medium to greenhouse potting mix (Sunshine mix number 1; Fison's, Canada) and were covered with beakers for the first few days after transplantation to prevent desiccation. Greenhouse day/night temperatures were 25±2/19°C under a 16 h photoperiod with supplemental lights to provide 150 μmol m^{-2} s^{-1} light intensity. Herbicide resistance of primary transformants and progeny was tested by a leaf painting assay and/or spraying with a 1000-fold dilution of the commercial herbicide Glufosinate 200™ (AgrEvo, NJ, USA) containing 20% ammonium glufosinate.

Example 16 – Detecting the Presence of SB109-TPSP and Bar Genes in Transformed Wheat Plants

[0079] A total of 35 putative transgenic wheat lines containing the plasmid pSB109-TPSP (that contains ABA stress-inducible promoter driving *TPSP* fusion gene) were successfully regenerated. One-month-old plants that were transferred to pots in the greenhouse were tested for phosphinothricin-based herbicide-resistance by painting the leaves using 0.5% Basta™ (Hoechst-Roussel, Agri-Vet Company, Somerville, NJ). The leaves remained green in 57% transgenic plants and showed

Basta-herbicide resistance, but in sensitive and non-transgenic control plants the leaves turned yellow. Integration of *TPSP* gene was confirmed by PCR analysis. Two sets of primers were designed from the *TPSP* gene (TPS1/TPS2, TPP1/TPP2) for PCR analysis of the genomic DNA. Out of the 20 plant DNA samples analyzed using either of the primer pairs, 9 plants showed the expected PCR product, confirming the presence of the transgene. Interestingly, most of the primary transformants appears to be phenotypically normal, unlike the other reports in dicots where multiple phenotypic alterations/pleiotropic effects were observed when trehalose gene(s) were expressed constitutively. This may be because of the regulated expression of trehalose biosynthetic gene in wheat.

Example 17 – Transgenic Wheat Plants are Salt-Stress Tolerant

[0080] Transgenic plants that harbor the TPSP gene were analyzed for salt tolerance. Leaf segments of 0.5 cm long were cut from transgenic and non-transgenic plants and floated on different solutions of NaCl (200, 400, and 800 mM) with the upper surface of the discs in contact with the solution and kept under continuous white light for 72 hours. The leaf segments were then rinsed with distilled water and extracted with DMF (N, N'-dimethyl formamide) by grinding with 1 ml of DMF with a pestle and mortar. The homogenate and washing solution (1 ml) with the solvent were centrifuged at 2,500 rpm for 10 minutes. The pellet was then vortexed with 0.5 ml of solvent and the pooled supernatants were adjusted to a final volume of 3 ml. The absorption (A) of the leaf extract at 664 nm and 647 nm was measured with a spectrophotometer. Chl-a, Chl-b, and Chl-a + Chl-b concentrations (μg/ml) were calculated by the following equations: Chl-a = 12.00 A-664 minus 3.11 A-647, Chl-b = 20.78 A-664 minus 4.88 A-647, and Chl-a + Chl-b = 17.67 A-647 + 7.12 A-664.

[0081] The results showed that leaf segments from the plants expressing the TPSP gene showed tolerance to NaCl with little or no significant bleaching, whereas that from the wild type showed extensive bleaching. Next, chlorophyll was isolated from control samples without salt treatment and samples after 72 hours of NaCl treatment. Chlorophyll content in plants in the absence of salt treatment was determined and set at 100. The results showed that in non-transgenic control plants,

the chlorophyll content was decreased by approximately 15% at 400 mM salt, and approximately 25% at 800 mM NaCl. In contrast, in the case of transgenic lines, after salt stress the chlorophyll content was almost as high as that without salt stress.

Example 18 – Transgenic Wheat Plants are Water-Stress Tolerant

[0082] A test for water-stress tolerance was carried out by measuring the electrolyte conductivity of the solution after soaking the leaf samples. Leaf segments were excised from plants. Duplicate samples (5 mg each) from each of two non-transgenic plants and each of four transgenic plants were excised from the plants. The leaf samples were placed on dry filter paper in 9-cm diameter Petri dishes and allowed to dry inside of a Laminar Flow Hood. Six hours later, the samples were transferred to different test-tubes that contained 2 ml distilled water. The test-tubes were subjected to vacuum three times at five-minute intervals at 60 psi to remove air bubbles adhered to the surface of leaves. The tubes then were shaken at 300 rpm for 2 hours in a slanted position. After shaking, the conductivity of the solution was measured using a conductivity meter (VWR International, West Chester, PA).

[0083] These results showed that the electrical conductivity of solutions used to soak leaves from non-transgenic plants was 5,400 μmho/mg leaf, whereas that from different transgenic lines was between 3,100 and 3,700 μmho/mg leaf. These results indicated that leaves from transgenic plants are less damaged by drying. In other words, leaves from transgenic plants are more tolerant to water stress.

[0084] Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.